

Express Mail No. EL610088706US

APPLICATION FOR UNITED STATES LETTERS PATENT

Applicants: Samuel I. Achilefu
Raghavan Rajagopalan
Richard B. Dorshow
Joseph E. Bugaj

Title: HYDROPHILIC CYANINE DYES

Assignee: Mallinckrodt Inc.

Beverly A. Lyman
WOOD, HERRON & EVANS, L.L.P.
2700 Carew Tower
Cincinnati OH 45202
(513) 241-2324

SPECIFICATION

HYDROPHILIC CYANINE DYES

This application is a continuation-in-part of application Serial No. 09/484,319, filed January 18, 2000.

Field of the Invention

5 This invention relates generally to compositions of cyanine dye bioconjugates with bioactive molecules for diagnosis and therapy, particularly, for visualization and detection of tumors.

Background of the Invention

10 Several dyes that absorb and emit light in the visible and near-infrared region of the electromagnetic spectrum are currently being used for various biomedical applications due to their biocompatibility, high molar absorptivity, and/or high fluorescence quantum yields. The high sensitivity of the optical modality in conjunction with dyes as contrast agents parallels that of nuclear medicine, and permits visualization of
15 organs and tissues without the undesirable effect of ionizing radiation.

Cyanine dyes with intense absorption and emission in the near-infrared (NIR) region are particularly useful because biological tissues

are optically transparent in this region (B. C. Wilson, Optical properties of tissues. *Encyclopedia of Human Biology*, 1991, 5, 587-597). For example, indocyanine green, which absorbs and emits in the NIR region, has been used for monitoring cardiac output, hepatic functions, and liver blood flow (Y-L. He, et al., Measurement of blood volume using indocyanine green measured with pulse-spectrometry: Its reproducibility and reliability. *Critical Care Medicine*, 1998, 26(8), 1446-1451; J. Caesar, et al., The use of Indocyanine green in the measurement of hepatic blood flow and as a test of hepatic function. *Clin. Sci.* 1961, 21, 43-57), and its functionalized derivatives have been used to conjugate biomolecules for diagnostic purposes (R. B. Mujumdar, et al., Cyanine dye labeling reagents: Sulfoindocyanine succinimidyl esters. *Bioconjugate Chemistry*, 1993, 4(2), 105-111; U.S. Patent No. 5,453,505; WO 98/48846; WO 98/22146; WO 96/17628; WO 98/48838).

A major drawback in the use of cyanine dye derivatives is the potential for hepatobiliary toxicity resulting from the rapid clearance of these dyes by the liver (G. R. Cherrick, et al., Indocyanine green: Observations on its physical properties, plasma decay, and hepatic extraction. *J. Clinical Investigation*, 1960, 39, 592-600). This is associated with the tendency of cyanine dyes in solution to form aggregates, which could be taken up by Kupffer cells in the liver.

Various attempts to obviate this problem have not been very successful. Typically, hydrophilic peptides, polyethyleneglycol or

oligosaccharide conjugates have been used, but these resulted in long-circulating products, which are eventually still cleared by the liver.

Another major difficulty with current cyanine and indocyanine dye systems is that they offer a limited scope in the ability to induce large

5 changes in the absorption and emission properties of these dyes.

Attempts have been made to incorporate various heteroatoms and cyclic moieties into the polyene chain of these dyes (L. Strekowski, et al.,

Substitution reactions of a nucleofugal group in heptamethine cyanine dyes. *J. Org. Chem.*, 1992, 57, 4578-4580; N. Narayanan and G.

10 Patonay, A new method for the synthesis of heptamethine cyanine dyes:

Synthesis of new near infrared fluorescent labels. *J. Org. Chem.*, 1995, 60, 2391-2395; U.S. Patent Nos. 5,732,104; 5,672,333; and

5,709,845), but the resulting dye systems do not show large differences in absorption and emission maxima, especially beyond 830 nm where

15 photoacoustic diagnostic applications are very sensitive. They also possess a prominent hydrophobic core, which enhances liver uptake.

Further, most cyanine dyes do not have the capacity to form starburst dendrimers, which are useful in biomedical applications.

For the purpose of tumor detection, many conventional dyes

20 are useful for *in vitro* applications because of their highly toxic effect on both normal and abnormal tissues. Other dyes lack specificity for particular organs or tissues and, hence, must be attached to bioactive carriers such as proteins, peptides, carbohydrates, and the like to deliver

the dyes to specific regions in the body. Several studies on the use of near infrared dyes and dye-biomolecule conjugates have been published (G. Patonay and M. D. Antoine, Near-Infrared Fluorogenic Labels: New Approach to an Old Problem, *Analytical Chemistry*, 1991, 63:321A-327A

- 5 and references therein; M. Brinkley, A Brief Survey of Methods for Preparing Protein Conjugates with Dyes, Haptens, and Cross-Linking Reagents, Perspectives in Bioconjugate Chemistry 1993, pp. 59-70, C. Meares (Ed), ACS Publication, Washington, DC; J. Slavik, Fluorescent Probes in Cellular and Molecular Biology, 1994, CRC Press, Inc.; U.S.
- 10 Patent No. 5,453,505; WO 98/48846; WO 98/22146; WO 96/17628; WO 98/48838).

Of particular interest is the targeting of tumor cells with antibodies or other large protein carriers such as transferrin as delivery vehicles (A. Becker et al., "Transferrin Mediated Tumor Delivery of Contrast Media for Optical Imaging and Magnetic Resonance Imaging", Biomedical Optics meeting, January 23-29, 1999, San Jose, CA). Such an approach has been widely used in nuclear medicine applications. Its major advantage is the retention of a carrier's tissue specificity, since the molecular volume of the dye is substantially smaller than the carrier.

- 15
- 20 However, this approach does have some serious limitations in that the diffusion of high molecular weight bioconjugates to tumor cells is highly unfavorable, and is further complicated by the net positive pressure in solid tumors (R. K. Jain, Barriers to Drug Delivery in Solid Tumors,

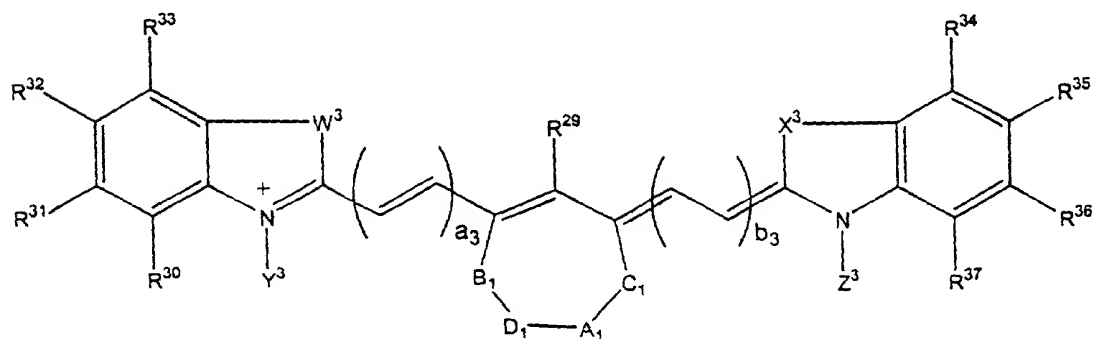
Scientific American 1994, 271:58-65. Furthermore, many dyes in general, and cyanine dyes in particular, tend to form aggregates in aqueous media that lead to fluorescence quenching.

Therefore, there is a need for dyes that could prevent dye aggregation in solution, that are predisposed to form dendrimers, that are capable of absorbing or emitting beyond 800 nm, that possess desirable photophysical properties, and that are endowed with tissue-specific targeting capability.

Summary of the Invention

The invention is directed to compositions, and methods of preparing the compositions, of low molecular weight biomolecule-dye conjugates to enhance tumor detection. The inventive compositions preserve the fluorescence efficiency of the dye molecules, do not aggregate in solution, form starburst dendrimers, are capable of absorbing or emitting light in the near infrared region (beyond 800 nm), and can be rendered tissue-specific.

In one embodiment, the inventive composition comprises cyanine dyes of general formula 1



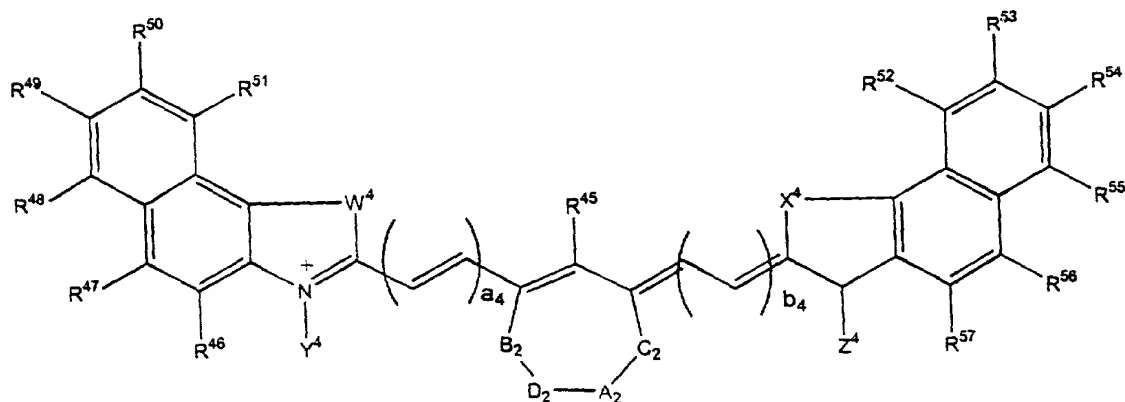
Formula 1

- wherein W^3 and X^3 may be the same or different and are selected from the group consisting of $-CR^1R^2$, $-O-$, $-NR^3$, $-S-$, and $-Se$; Y^3 is selected from the group consisting of $-(CH_2)_a-CONH-Bm$, $-CH_2-(CH_2OCH_2)_b-CH_2-CONH-Bm$, $-(CH_2)_a-NHCO-Bm$, $-CH_2-(CH_2OCH_2)_b-CH_2-NHCO-Bm$, $-(CH_2)_a-N(R^3)-(CH_2)_b-CONH-Bm$, $(CH_2)_a-N(R^3)-(CH_2)_c-NHCO-Bm$, $-(CH_2)_a-N(R^3)-CH_2-(CH_2OCH_2)_b-CH_2-CONH-Bm$, $-(CH_2)_a-N(R^3)-CH_2-(CH_2OCH_2)_b-CH_2-NHCO-Bm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-(CH_2)_a-CONH-Bm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-(CH_2)_a-NHCO-Bm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-CH_2-(CH_2OCH_2)_d-CONH-Bm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-CH_2-(CH_2OCH_2)_d-NHCO-Bm$, $-(CH_2)_a-NR^3R^4$, and $-CH_2(CH_2OCH_2)_b-CH_2NR^3R^4$; Z^3 is selected from the group consisting of $-(CH_2)_a-CONH-Dm$, $-CH_2-(CH_2OCH_2)_b-CH_2-CONH-Dm$, $-(CH_2)_a-NHCO-Dm$, $-CH_2-(CH_2OCH_2)_b-CH_2-NHCO-Dm$, $-(CH_2)_a-N(R^3)-(CH_2)_b-CONH-Dm$, $(CH_2)_a-N(R^3)-(CH_2)_c-NHCO-Dm$, $-(CH_2)_a-N(R^3)-CH_2-(CH_2OCH_2)_b-CH_2-CONH-Dm$, $-(CH_2)_a-N(R^3)-CH_2-(CH_2OCH_2)_b-CH_2-NHCO-Dm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-(CH_2)_a-CONH-Dm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-(CH_2)_a-NHCO-Dm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-CH_2-(CH_2OCH_2)_d-CONH-Dm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-CH_2-(CH_2OCH_2)_d-NHCO-Dm$, $-(CH_2)_a-NR^3R^4$, and $-CH_2(CH_2OCH_2)_b-CH_2NR^3R^4$; A_1 is a single or a double bond; B_1 , C_1 , and D_1 may be the same or different and are selected from the group consisting of $-O-$, $-S-$, $-Se-$, $-P-$, $-CR^1R^2$, $-CR^1$, alkyl, NR^3 , and $-C=O$; A_1 , B_1 , C_1 , and D_1 may together form a 6- to 12-membered carbocyclic ring or a 6- to 12-membered heterocyclic ring optionally containing one or more oxygen, nitrogen, or sulfur atom; a_3 and b_3 are independently from 0 to 5;

R^1 to R^4 , and R^{29} to R^{37} are independently selected from the group consisting of hydrogen, C_1 - C_{10} alkyl, C_5 - C_{20} aryl, C_1 - C_{10} alkoxy, C_1 - C_{10} polyalkoxyalkyl, C_1 - C_{20} polyhydroxyalkyl, C_5 - C_{20} polyhydroxyaryl, C_1 - C_{10} aminoalkyl, cyano, nitro, halogen, saccharide, peptide, $-\text{CH}_2(\text{CH}_2\text{OCH}_2)_b-$
5 $\text{CH}_2\text{-OH}$, $-(\text{CH}_2)_a\text{-CO}_2\text{H}$, $-(\text{CH}_2)_a\text{-CONH-Bm}$, $-\text{CH}_2\text{-(CH}_2\text{OCH}_2)_b\text{-CH}_2\text{-CONH-Bm}$, $-(\text{CH}_2)_a\text{-NHCO-Bm}$, $-\text{CH}_2\text{-(CH}_2\text{OCH}_2)_b\text{-CH}_2\text{-NHCO-Bm}$, $-(\text{CH}_2)_a\text{-OH}$ and $-\text{CH}_2\text{-(CH}_2\text{OCH}_2)_b\text{-CO}_2\text{H}$; Bm and Dm are independently selected from the group consisting of a bioactive peptide, a protein, a cell, an antibody, an antibody fragment, a saccharide, a glycopeptide, a peptidomimetic, a
10 drug, a drug mimic, a hormone, a metal chelating agent, a radioactive or nonradioactive metal complex, and an echogenic agent; a and c are independently from 1 to 20; and b and d are independently from 1 to 100.

In a second embodiment, the inventive composition

15 comprises cyanine dyes of general formula 2

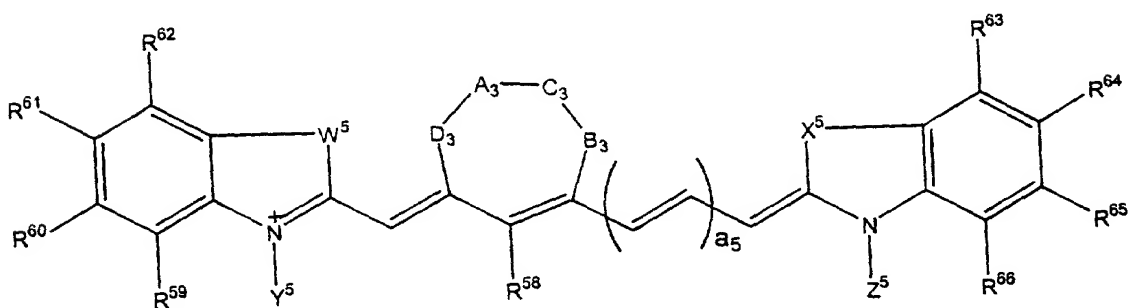


Formula 2

- wherein W^4 and X^4 may be the same or different and are selected from the group consisting of $-CR^1R^2$, $-O-$, $-NR^3$, $-S-$, and $-Se$; Y^4 is selected from the group consisting of $-(CH_2)_a-CONH-Bm$, $-CH_2-(CH_2OCH_2)_b-CH_2-CONH-Bm$, $-(CH_2)_a-NHCO-Bm$, $-CH_2-(CH_2OCH_2)_b-CH_2-NHCO-Bm$, $-(CH_2)_a-N(R^3)-(CH_2)_b-CONH-Bm$, $(CH_2)_a-N(R^3)-(CH_2)_c-NHCO-Bm$, $-(CH_2)_a-N(R^3)-CH_2-(CH_2OCH_2)_b-CH_2-CONH-Bm$, $-(CH_2)_a-N(R^3)-CH_2-(CH_2OCH_2)_b-CH_2-NHCO-Bm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-(CH_2)_a-CONH-Bm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-(CH_2)_a-NHCO-Bm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-CH_2-(CH_2OCH_2)_d-CONH-Bm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-CH_2-(CH_2OCH_2)_d-NHCO-Bm$, $-(CH_2)_a-NR^3R^4$, and $-CH_2(CH_2OCH_2)_b-CH_2NR^3R^4$; Z^4 is selected from the group consisting of $-(CH_2)_a-CONH-Dm$, $-CH_2-(CH_2OCH_2)_b-CH_2-CONH-Dm$, $-(CH_2)_a-NHCO-Dm$, $-CH_2-(CH_2OCH_2)_b-CH_2-NHCO-Dm$, $-(CH_2)_a-N(R^3)-(CH_2)_b-CONH-Dm$, $(CH_2)_a-N(R^3)-(CH_2)_c-NHCO-Dm$, $-(CH_2)_a-N(R^3)-CH_2-(CH_2OCH_2)_b-CH_2-CONH-Dm$, $-(CH_2)_a-N(R^3)-CH_2-(CH_2OCH_2)_b-CH_2-NHCO-Dm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-(CH_2)_a-CONH-Dm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-(CH_2)_a-NHCO-Dm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-CH_2-(CH_2OCH_2)_d-CONH-Dm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-CH_2-(CH_2OCH_2)_d-NHCO-Dm$, $-(CH_2)_a-NR^3R^4$, and $-CH_2(CH_2OCH_2)_b-CH_2NR^3R^4$; A_2 is a single or a double bond; B_2 , C_2 , and D_2 may be the same or different and are selected from the group consisting of $-O-$, $-S-$, $-Se-$, $-P-$, $-CR^1R^2$, $-CR^1$, alkyl, NR^3 , and $-C=O$; A_2 , B_2 , C_2 , and D_2 may together form a 6- to 12-membered carbocyclic ring or a 6- to 12-membered heterocyclic ring optionally containing one or more oxygen, nitrogen, or sulfur atom; a_4 and b_4 independently vary from 0 to

5; R^1 to R^4 , and R^{45} to R^{57} are independently selected from the group consisting of hydrogen, C_1 - C_{10} alkyl, C_5 - C_{20} aryl, C_1 - C_{10} alkoxy, C_1 - C_{10} polyalkoxyalkyl, C_1 - C_{20} polyhydroxyalkyl, C_5 - C_{20} polyhydroxyaryl, C_1 - C_{10} aminoalkyl, cyano, nitro, halogen, saccharide, peptide, $-\text{CH}_2(\text{CH}_2\text{OCH}_2)_b-$
 5 $\text{CH}_2\text{-OH}$, $-(\text{CH}_2)_a\text{-CO}_2\text{H}$, $-(\text{CH}_2)_a\text{-CONH-Bm}$, $-\text{CH}_2-(\text{CH}_2\text{OCH}_2)_b\text{-CH}_2\text{-CONH-Bm}$, $-(\text{CH}_2)_a\text{-NHCO-Bm}$, $-\text{CH}_2-(\text{CH}_2\text{OCH}_2)_b\text{-CH}_2\text{-NHCO-Bm}$, $-(\text{CH}_2)_a\text{-OH}$ and $-\text{CH}_2-(\text{CH}_2\text{OCH}_2)_b\text{-CO}_2\text{H}$; Bm and Dm are independently selected from the group consisting of a bioactive peptide, a protein, a cell, an antibody, an antibody fragment, a saccharide, a glycopeptide, a peptidomimetic, a
 10 drug, a drug mimic, a hormone, a metal chelating agent, a radioactive or nonradioactive metal complex, and an echogenic agent; a and c are independently from 1 to 20; and b and d are independently from 1 to 100.

In a third embodiment, the inventive composition comprises
 15 cyanine dyes of general formula 3



Formula 3

wherein W^5 and X^5 may be the same or different and are selected from the group consisting of $-CR^1R^2$, $-O-$, $-NR^3$, $-S-$, and $-Se$; Y^5 is selected from the group consisting of $-(CH_2)_a-CONH-Bm$, $-CH_2-(CH_2OCH_2)_b-CH_2-CONH-Bm$, $-(CH_2)_a-NHCO-Bm$, $-CH_2-(CH_2OCH_2)_b-CH_2-NHCO-Bm$, $-(CH_2)_a-N(R^3)-(CH_2)_b-CONH-Bm$, $(CH_2)_a-N(R^3)-(CH_2)_c-NHCO-Bm$, $-(CH_2)_a-N(R^3)-CH_2-(CH_2OCH_2)_b-CH_2-CONH-Bm$, $-(CH_2)_a-N(R^3)-CH_2-(CH_2OCH_2)_b-CH_2-NHCO-Bm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-(CH_2)_a-CONH-Bm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-(CH_2)_a-NHCO-Bm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-CH_2-(CH_2OCH_2)_d-CONH-Bm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-CH_2-(CH_2OCH_2)_d-NHCO-Bm$, $-(CH_2)_a-NR^3R^4$, and $-CH_2(CH_2OCH_2)_b-CH_2NR^3R^4$; Z^5 is selected from the group consisting of $-(CH_2)_a-CONH-Dm$, $-CH_2-(CH_2OCH_2)_b-CH_2-CONH-Dm$, $-(CH_2)_a-NHCO-Dm$, $-CH_2-(CH_2OCH_2)_b-CH_2-NHCO-Dm$, $-(CH_2)_a-N(R^3)-(CH_2)_b-CONH-Dm$, $(CH_2)_a-N(R^3)-(CH_2)_c-NHCO-Dm$, $-(CH_2)_a-N(R^3)-CH_2-(CH_2OCH_2)_b-CH_2-CONH-Dm$, $-(CH_2)_a-N(R^3)-CH_2-(CH_2OCH_2)_b-CH_2-NHCO-Dm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-(CH_2)_a-CONH-Dm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-(CH_2)_a-NHCO-Dm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-CH_2-(CH_2OCH_2)_d-CONH-Dm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-CH_2-(CH_2OCH_2)_d-NHCO-Dm$, $-(CH_2)_a-NR^3R^4$, and $-CH_2(CH_2OCH_2)_b-CH_2NR^3R^4$; A_3 is a single or a double bond; B_3 , C_3 , and D_3 may be the same or different and are selected from the group consisting of $-O-$, $-S-$, $-Se-$, $-P-$, $-CR^1R^2$, $-CR^1$, alkyl, NR^3 , and $-C=O$; A_3 , B_3 , C_3 , and D_3 may together form a 6- to 12-membered carbocyclic ring or a 6- to 12-membered heterocyclic ring optionally containing one or more oxygen, nitrogen, or sulfur atom; a_5 is independently from 0 to 5; R^1 to

R⁴, and R⁵⁸ to R⁶⁶ are independently selected from the group consisting of hydrogen, C₁-C₁₀ alkyl, C₅-C₂₀ aryl, C₁-C₁₀ alkoxy, C₁-C₁₀ polyalkoxyalkyl, C₁-C₂₀ polyhydroxyalkyl, C₅-C₂₀ polyhydroxyaryl, C₁-C₁₀ aminoalkyl, cyano, nitro, halogen, saccharide, peptide, -CH₂(CH₂OCH₂)_b-CH₂-OH, -(CH₂)_a-

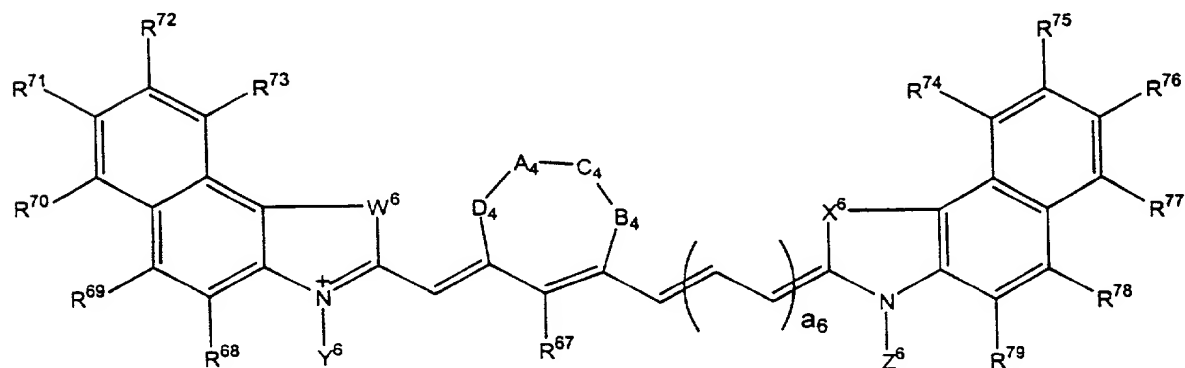
5 CO₂H, -(CH₂)_a-CONH-Bm, -CH₂-(CH₂OCH₂)_b-CH₂-CONH-Bm, -(CH₂)_a-NHCO-Bm, -CH₂-(CH₂OCH₂)_b-CH₂-NHCO-Bm, -(CH₂)_a-OH and -CH₂-(CH₂OCH₂)_b-

CO₂H; Bm and Dm are independently selected from the group consisting of a bioactive peptide, a protein, a cell, an antibody, an antibody

fragment, a saccharide, a glycopeptide, a peptidomimetic, a drug, a drug

10 mimic, a hormone, a metal chelating agent, a radioactive or nonradioactive metal complex, and an echogenic agent; a and c are independently from 1 to 20; and b and d are independently from 1 to 100.

In a fourth embodiment, inventive composition comprises cyanine dyes of general formula 4



Formula 4

wherein W^6 and X^6 may be the same or different and are selected from the group consisting of $-CR^1R^2$, $-O-$, $-NR^3$, $-S-$, and $-Se$; Y^6 is selected from the group consisting of $-(CH_2)_a-CONH-Bm$, $-CH_2-(CH_2OCH_2)_b-CH_2-CONH-Bm$, $-(CH_2)_a-NHCO-Bm$, $-CH_2-(CH_2OCH_2)_b-CH_2-NHCO-Bm$, $-(CH_2)_a-N(R^3)-(CH_2)_b-CONH-Bm$, $(CH_2)_a-N(R^3)-(CH_2)_c-NHCO-Bm$, $-(CH_2)_a-N(R^3)-CH_2-(CH_2OCH_2)_b-CH_2-CONH-Bm$, $-(CH_2)_a-N(R^3)-CH_2-(CH_2OCH_2)_b-CH_2-NHCO-Bm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-(CH_2)_a-CONH-Bm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-(CH_2)_a-NHCO-Bm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-CH_2-(CH_2OCH_2)_d-CONH-Bm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-CH_2-(CH_2OCH_2)_d-NHCO-Bm$, $-(CH_2)_a-NR^3R^4$, and $-CH_2(CH_2OCH_2)_b-CH_2NR^3R^4$; Z^6 is selected from the group consisting of $-(CH_2)_a-CONH-Dm$, $-CH_2-(CH_2OCH_2)_b-CH_2-CONH-Dm$, $-(CH_2)_a-NHCO-Dm$, $-CH_2-(CH_2OCH_2)_b-CH_2-NHCO-Dm$, $-(CH_2)_a-N(R^3)-(CH_2)_b-CONH-Dm$, $(CH_2)_a-N(R^3)-(CH_2)_c-NHCO-Dm$, $-(CH_2)_a-N(R^3)-CH_2-(CH_2OCH_2)_b-CH_2-CONH-Dm$, $-(CH_2)_a-N(R^3)-CH_2-(CH_2OCH_2)_b-CH_2-NHCO-Dm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-(CH_2)_a-CONH-Dm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-(CH_2)_a-NHCO-Dm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-CH_2-(CH_2OCH_2)_d-CONH-Dm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-CH_2-(CH_2OCH_2)_d-NHCO-Dm$, $-(CH_2)_a-NR^3R^4$, and $-CH_2(CH_2OCH_2)_b-CH_2NR^3R^4$; A_4 is a single or a double bond; B_4 , C_4 , and D_4 may be the same or different and are selected from the group consisting of $-O-$, $-S-$, $-Se-$, $-P-$, $-CR^1R^2$, $-CR^1$, alkyl, NR^3 , and $-C=O$; A_4 , B_4 , C_4 , and D_4 may together form a 6- to 12-membered carbocyclic ring or a 6- to 12-membered heterocyclic ring optionally containing one or more oxygen, nitrogen, or sulfur atom; a_6 is independently from 0 to 5; R^1 to

R⁴, and R⁶⁷ to R⁷⁹ are independently selected from the group consisting of hydrogen, C₁-C₁₀ alkyl, C₅-C₂₀ aryl, C₁-C₁₀ alkoxy, C₁-C₁₀ polyalkoxyalkyl, C₁-C₂₀ polyhydroxyalkyl, C₅-C₂₀ polyhydroxyaryl, C₁-C₁₀ aminoalkyl, cyano, nitro, halogen, saccharide, peptide, -CH₂(CH₂OCH₂)_b-CH₂-OH, -(CH₂)_a-CO₂H, -(CH₂)_a-CONH-Bm, -CH₂-(CH₂OCH₂)_b-CH₂-CONH-Bm, -(CH₂)_a-NHCO-Bm, -CH₂-(CH₂OCH₂)_b-CH₂-NHCO-Bm, -(CH₂)_a-OH or -CH₂-(CH₂OCH₂)_b-CO₂H; Bm and Dm are independently selected from the group consisting of a bioactive peptide, a protein, a cell, an antibody, an antibody fragment, a saccharide, a glycopeptide, a peptidomimetic, a drug, a drug mimic, a hormone, a metal chelating agent, a radioactive and nonradioactive metal complex, and an echogenic agent; a and c are independently from 1 to 20; and b and d are independently from 1 to 100.

The invention will be further appreciated in light of the following figures, detailed description, and examples.

Brief Description of the Figures

The file of this patent contains at least one drawing executed in color. Copies of this patent with color drawing(s) will be provided by the Patent and Trademark Office upon request and payment of the necessary fee.

FIG. 1 shows the reaction pathway for the synthesis of bis-carboxylic acid cyanine dyes.

FIG. 2 shows the reaction pathway for the synthesis of tetracarboxylic acid cyanine dyes.

FIG. 3 shows the reaction pathway for the synthesis of polyhydroxycarboxylic acid dyes.

5 FIG. 4 shows the reaction pathway for the synthesis of non-aggregating cyanine dyes.

FIG. 5 shows the reaction pathway for the synthesis of long wavelength absorbing dyes.

FIG. 6 shows the reaction pathway for the synthesis of
10 cyanine dye bioconjugates.

FIGS. 7A-F represent images at 2 minutes and 30 minutes post injection of indocyanine green (ICG) into rats with various tumors.

FIGS. 8A-B show a comparison of the uptake of ICG
(FIG. 8A) and Cytate 1 (FIG. 8B) in rats with the pancreatic acinar
15 carcinoma (CA20948).

FIGS. 9A-B show images of rats with the pancreatic acinar carcinoma (CA20948) 45 minutes (FIG. 9A) and 27 hours (FIG. 9B) post injection of Cytate 1.

FIG. 10 is an image of individual organs taken from a rat with
20 pancreatic acinar carcinoma (CA20948) about 24 hours after injection with Cytate 1.

FIG. 11 is an image of bombesinate in an AR42-J tumor-bearing rat 22 hours after injection.

FIG. 12 is the clearance profile of Cytate 1 from the blood of a normal rat.

FIG. 13 is the clearance profile of Cytate 1 from the blood of a pancreatic tumor-bearing rat.

5 FIG. 14 is the clearance profile of Cytate 2 from the blood of a normal rat.

FIG. 15 is the clearance profile of Cytate 2 from the blood of a pancreatic tumor-bearing rat.

10 FIG. 16 is the clearance profile of Cytate 4 from the blood of a normal rat.

Detailed Description of the Invention

The novel compositions of the present invention comprising dyes of formulas 1 to 4 offer significant advantages over those currently described in the art. These inventive dyes form starburst dendrimers

15 which prevent aggregation in solution by preventing intramolecular and intermolecular ordered hydrophobic interactions, and have multiple attachment sites proximal to the dye chromophore for ease of forming bioactive molecules. The presence of rigid and extended chromophore backbone enhances their fluorescence quantum yield and extends their

20 maximum absorption beyond 800 nm. Conjugation of biomolecules to these dyes is readily achievable.

The inventive bioconjugates of the present invention also exploit the symmetric nature of the cyanine and indocyanine dye

structures by incorporating one to ten receptor targeting groups in close proximity to each other, such that the receptor binding can be greatly enhanced due to a cooperative effect. Accordingly, several cyanine dyes containing one or more targeting domains have been prepared and tested *in vivo* for biological activity.

The inventive dye-bioconjugates of formulas 1 to 4 are useful for various biomedical applications. These include, but are not limited to, tomographic imaging of organs, monitoring of organ functions, coronary angiography, fluorescence endoscopy, detection, imaging, and therapy of tumors, laser assisted guided surgery, photoacoustic methods, and sonofluorescent methods.

Specific embodiments to accomplish some of the aforementioned biomedical applications are given below. The novel dyes of the present invention are prepared according the methods well known in the art and are illustrated in FIGS. 1-5.

FIG. 1 illustrates the synthetic scheme for bis-carboxylic acid cyanine dyes, where $A = CH_2$ or CH_2OCH_2 ; $R = COOH$; $R' = COOH$, $NHfmoc$; CO_2t-Bu ; SO_3^- ; $R_1 = R_2 = H$ (Formula 1) or $R_1, R_2 =$ fused phenyl (Formula 2).

FIG. 2 illustrates the synthetic scheme for tetracarboxylic acid cyanine dyes, where $A = CH_2$ or CH_2OCH_2 ; $R_1 = R_2 = H$ (Formula 1) or $R_1, R_2 =$ fused phenyl (Formula 2).

FIG. 3 illustrates the synthetic scheme for polyhydroxy-carboxylic acid cyanine dyes.

FIG. 4 illustrates the synthetic scheme for non-aggregating cyanine dyes.

5 FIG. 5 illustrates the synthetic scheme for long wavelength-absorbing tunable cyanine dyes.

In one embodiment, the inventive bioconjugates have the Formula 1, wherein W^3 and X^3 may be the same or different and are selected from the group consisting of $-C(CH_3)_2$, $-C((CH_2)_aOH)CH_3$,
 10 $-C((CH_2)_aOH)_2$, $-C((CH_2)_aCO_2H)CH_3$, $-C((CH_2)_aCO_2H)_2$,
 $-C((CH_2)_aNH_2)CH_3$, $-C((CH_2)_aNH_2)_2$, $-C((CH_2)_aNR^3R^4)_2$, $-NR^3$, and $-S$; Y^3 is selected from the group consisting of $-(CH_2)_a-CONH-Bm$, $-CH_2$
 $-(CH_2OCH_2)_b-CH_2-CONH-Bm$, $-(CH_2)_a-NHCO-Bm$, $-CH_2-(CH_2OCH_2)_b-CH_2$
 $-NHCO-Bm$, $-(CH_2)_a-NR^3R^4$, and $-CH_2(CH_2OCH_2)_b-CH_2NR^3R^4$; Z^3 is selected
 15 from the group consisting of $-(CH_2)_a-CONH-Dm$, $-CH_2-(CH_2OCH_2)_b-CH_2$
 $-CONH-Dm$, $-(CH_2)_a-NHCO-Dm$, $-CH_2-(CH_2OCH_2)_b-CH_2-NHCO-Dm$, $-(CH_2)_a$
 $-NR^3R^4$, and $-CH_2(CH_2OCH_2)_b-CH_2NR^3R^4$; A_1 is a single or a double bond;
 B_1 , C_1 , and D_1 are independently selected from the group consisting of
 $-O-$, $-S-$, NR^3 , $(CH_2)_a-CR^1R^2$, and $-CR^1$; A_1 , B_1 , C_1 , and D_1 may together
 20 form a 6- to 10-membered carbocyclic ring or a 6- to 10-membered heterocyclic ring optionally containing one or more oxygen, nitrogen, or sulfur atom; a_3 and b_3 are independently from 0 to 3; R^1 to R^4 , and R^{29} to R^{37} are independently selected from the group consisting of hydrogen, C_1

-C₁₀ alkyl, C₅-C₁₂ aryl, C₁-C₁₀ alkoxy, C₁-C₁₀ polyhydroxyalkyl, C₅-C₁₂ polyhydroxyaryl, C₁-C₁₀ aminoalkyl, mono- or oligosaccharide, peptide with 2 to 30 amino acid units, -CH₂(CH₂OCH₂)_b-CH₂-OH, -(CH₂)_a-CO₂H, -(CH₂)_a-CONH-Bm, -CH₂-(CH₂OCH₂)_b-CH₂-CONH-Bm, -(CH₂)_a-NHCO-Bm, 5 -CH₂-(CH₂OCH₂)_b-CH₂-NHCO-Bm, -(CH₂)_a-OH and -CH₂-(CH₂OCH₂)_b-CO₂H; Bm and Dm are independently selected from the group consisting of a bioactive peptide containing 2 to 30 amino acid units, an antibody, a mono- or oligosaccharide, a glycopeptide, a metal chelating agent, a radioactive or nonradioactive metal complex, and an echogenic agent; a 10 and c are independently from 1 to 10; and b and d are independently from 1 to 30.

In a second embodiment, the inventive bioconjugates have the general Formula 2, wherein W⁴ and X⁴ may be the same or different and are selected from the group consisting of -C(CH₃)₂, -C((CH₂)_aOH)CH₃, 15 -C((CH₂)_aOH)₂, -C((CH₂)_aCO₂H)CH₃, -C((CH₂)_aCO₂H)₂, -C((CH₂)_aNH₂)CH₃, C((CH₂)_aNH₂)₂, -C((CH₂)_aNR³R⁴)₂, -NR³, and -S-; Y⁴ is selected from the group consisting of -(CH₂)_a-CONH-Bm, -CH₂-(CH₂OCH₂)_b-CH₂-CONH-Bm, -(CH₂)_a-NHCO-Bm, -CH₂-(CH₂OCH₂)_b-CH₂-NHCO-Bm, -(CH₂)_a-NR³R⁴, and 20 -CH₂-(CH₂OCH₂)_b-CH₂NR³R⁴; Z⁴ is selected from the group consisting of -(CH₂)_a-CONH-Dm, -CH₂-(CH₂OCH₂)_b-CH₂-CONH-Dm, -(CH₂)_a-NHCO-Dm, -CH₂-(CH₂OCH₂)_b-CH₂-NHCO-Dm, -(CH₂)_a-NR³R⁴, and

$-\text{CH}_2(\text{CH}_2\text{OCH}_2)_b-\text{CH}_2\text{NR}^3\text{R}^4$; A_2 is a single or a double bond; B_2 , C_2 , and D_2

are independently selected from the group consisting of

$-\text{O}-$, $-\text{S}-$, NR^3 , $(\text{CH}_2)_a-\text{CR}^1\text{R}^2$, and $-\text{CR}^1$; A_2 , B_2 , C_2 , and D_2 may together

form a 6- to 10-membered carbocyclic ring or a 6- to 10-membered

- 5 heterocyclic ring optionally containing one or more oxygen, nitrogen, or sulfur atom; a_4 and b_4 are independently from 0 to 3; R^1 to R^4 , and R^{45} to R^{57} are independently selected from the group consisting of hydrogen,

C_1 - C_{10} alkyl, C_5 - C_{12} aryl, C_1 - C_{10} alkoxy, C_1 - C_{10} polyhydroxyalkyl, C_5 - C_{12} polyhydroxyaryl, C_1 - C_{10} aminoalkyl, mono- or oligosaccharide, peptide

- 10 with 2 to 30 amino acid units, $-\text{CH}_2(\text{CH}_2\text{OCH}_2)_b-\text{CH}_2\text{-OH}$, $-(\text{CH}_2)_a-\text{CO}_2\text{H}$, $-(\text{CH}_2)_a-\text{CONH-Bm}$, $-\text{CH}_2-(\text{CH}_2\text{OCH}_2)_b-\text{CH}_2-\text{CONH-Bm}$, $-(\text{CH}_2)_a-\text{NHCO-Bm}$, $-\text{CH}_2-(\text{CH}_2\text{OCH}_2)_b-\text{CH}_2-\text{NHCO-Bm}$, $-(\text{CH}_2)_a-\text{OH}$ and $-\text{CH}_2-(\text{CH}_2\text{OCH}_2)_b-\text{CO}_2\text{H}$;

Bm and Dm are independently selected from the group consisting of a

bioactive peptide containing 2 to 30 amino acid units, an antibody, a

- 15 mono- or oligosaccharide, a glycopeptide, a metal chelating agent, a radioactive or nonradioactive metal complex, and an echogenic agent; a and c are independently from 1 to 10; and b and d are independently from 1 to 30.

In a third embodiment, the inventive bioconjugates have the

- 20 general Formula 3, wherein W^5 and X^5 may be the same or different and are selected from the group consisting of $-\text{C}(\text{CH}_3)_2$, $-\text{C}((\text{CH}_2)_a\text{OH})\text{CH}_3$, $-\text{C}((\text{CH}_2)_a\text{OH})_2$, $-\text{C}((\text{CH}_2)_a\text{CO}_2\text{H})\text{CH}_3$, $-\text{C}((\text{CH}_2)_a\text{CO}_2\text{H})_2$,

- C((CH₂)_aNH₂)CH₃, -C((CH₂)_aNH₂)₂, -C((CH₂)_aNR³R⁴)₂, -NR³, and -S-; Y⁵ is selected from the group consisting of -(CH₂)_a-CONH-Bm, -CH₂-(CH₂OCH₂)_b-CH₂-CONH-Bm, -(CH₂)_a-NHCO-Bm, -CH₂-(CH₂OCH₂)_b-CH₂-NHCO-Bm, -(CH₂)_a-NR³R⁴, and
- 5 -CH₂(CH₂OCH₂)_b-CH₂NR³R⁴; Z⁵ is selected from the group consisting of -(CH₂)_a-CONH-Dm, -CH₂-(CH₂OCH₂)_b-CH₂-CONH-Dm, -(CH₂)_a-NHCO-Dm, -CH₂-(CH₂OCH₂)_b-CH₂-NHCO-Dm, -(CH₂)_a-NR³R⁴, and -CH₂(CH₂OCH₂)_b-CH₂NR³R⁴; A₃ is a single or a double bond; B₃, C₃, and D₃ are independently selected from the group consisting of
- 10 -O-, -S-, NR³, (CH₂)_a-CR¹R², and -CR¹; A₃, B₃, C₃, and D₃ may together form a 6- to 10-membered carbocyclic ring or a 6- to 10-membered heterocyclic ring optionally containing one or more oxygen, nitrogen, or sulfur atom; a₅ is from 0 to 3; R¹ to R⁴, and R⁵⁸ to R⁶⁶ are independently selected from the group consisting of hydrogen, C₁-C₁₀ alkyl, C₅-C₁₂ aryl,
- 15 C₁-C₁₀ alkoxy, C₁-C₁₀ polyhydroxyalkyl, C₅-C₁₂ polyhydroxyaryl, C₁-C₁₀ aminoalkyl, mono- or oligosaccharide, peptide with 2 to 30 amino acid units, -CH₂(CH₂OCH₂)_b-CH₂-OH, -(CH₂)_a-CO₂H, -(CH₂)_a-CONH-Bm, -CH₂-(CH₂OCH₂)_b-CH₂-CONH-Bm, -(CH₂)_a-NHCO-Bm, -CH₂-(CH₂OCH₂)_b-CH₂-NHCO-Bm, -(CH₂)_a-OH and -CH₂-(CH₂OCH₂)_b-CO₂H;
- 20 Bm and Dm are independently selected from the group consisting of a bioactive peptide containing 2 to 30 amino acid units, an antibody, a mono- or oligosaccharide, a glycopeptide, a metal chelating agent, a radioactive or nonradioactive metal complex, and an echogenic agent; a

and c are independently from 1 to 10; and b and d are independently from 1 to 30.

In a fourth embodiment, the inventive bioconjugates have the general Formula 4, wherein W⁶ and X⁶ may be the same or different and

- 5 are selected from the group consisting of -C(CH₃)₂, -C((CH₂)_aOH)CH₃, -C((CH₂)_aOH)₂, -C((CH₂)_aCO₂H)CH₃, -C((CH₂)_aCO₂H)₂, -C((CH₂)_aNH₂)CH₃, -C((CH₂)_aNH₂)₂, C((CH₂)_aNR³R⁴)₂, -NR³, and -S-; Y⁶ is selected from the group consisting of -(CH₂)_a-CONH-Bm, -CH₂-(CH₂OCH₂)_b-CH₂-CONH-Bm, -(CH₂)_a-NHCO-Bm,
- 10 -CH₂-(CH₂OCH₂)_b-CH₂-NHCO-Bm, -(CH₂)_a-NR³R⁴, and -CH₂(CH₂OCH₂)_b-CH₂NR³R⁴; Z⁶ is selected from the group consisting of -(CH₂)_a-CONH-Dm, -CH₂-(CH₂OCH₂)_b-CH₂-CONH-Dm, -(CH₂)_a-NHCO-Dm, -CH₂-(CH₂OCH₂)_b-CH₂-NHCO-Dm, -(CH₂)_a-NR³R⁴, and -CH₂(CH₂OCH₂)_b-CH₂NR³R⁴; A₄ is a single or a double bond; B₄, C₄, and D₄
- 15 are independently selected from the group consisting of -O-, -S-, NR³, (CH₂)_a-CR¹R², and -CR¹; A₄, B₄, C₄, and D₄ may together form a 6- to 10-membered carbocyclic ring or a 6- to 10-membered heterocyclic ring optionally containing one or more oxygen, nitrogen, or sulfur atom; a₆ is from 0 to 3; R¹ to R⁴, and R⁶⁷ to R⁷⁹ are independently selected from the
- 20 group consisting of hydrogen, C₁-C₁₀ alkyl, C₅-C₁₂ aryl, C₁-C₁₀ alkoxy, C₁-C₁₀ polyhydroxyalkyl, C₅-C₁₂ polyhydroxyaryl, C₁-C₁₀ aminoalkyl, mono- or oligosaccharide, peptide with 2 to 30 amino acid units, -CH₂(CH₂OCH₂)_b-CH₂-OH, -(CH₂)_a-CO₂H, -(CH₂)_a-CONH-Bm,

$-\text{CH}_2-(\text{CH}_2\text{OCH}_2)_b-\text{CH}_2-\text{CONH-Bm}$, $-(\text{CH}_2)_a-\text{NHCO-Bm}$,
 $-\text{CH}_2-(\text{CH}_2\text{OCH}_2)_b-\text{CH}_2-\text{NHCO-Bm}$, $-(\text{CH}_2)_a-\text{OH}$ and $-\text{CH}_2-(\text{CH}_2\text{OCH}_2)_b-\text{CO}_2\text{H}$;

Bm and Dm are independently selected from the group consisting of a
bioactive peptide containing 2 to 30 amino acid units, an antibody, a

- 5 mono- or oligosaccharide, a glycopeptide, a metal chelating agent, a
radioactive or nonradioactive metal complex, and an echogenic agent; a
and c are independently from 1 to 10; and b and d are independently from
1 to 30.

- This invention is also related to the method of conjugating
- 10 the inventive dyes to peptides or biomolecules by solid phase or solution
synthesis methods. FIG. 6 illustrates the synthetic scheme for
bioconjugates incorporating the cyanine dyes of FIGS. 1-5, using
automated peptide synthesis in a solid support, where $\text{A} = \text{CH}_2$ or
 CH_2OCH_2 ; $\text{R}_1 = \text{R}_2 = \text{H}$ (Formula 1) or $\text{R}_1, \text{R}_2 = \text{fused phenyl}$ (Formula 2);
- 15 $\text{AA} = \text{amino acids}$; $\text{R} = \text{CONH peptide}$; $\text{R}' = \text{R}$ (bis conjugate) or COOH
(mono conjugate); $\text{P} = \text{solid support}$; $\text{P} = \text{presence or absence depends}$
on R' definition.

- This invention is also related to the method of preventing
fluorescence quenching. It is known that cyanine dyes generally form
- 20 aggregates in aqueous media, leading to fluorescence quenching. Where
the presence of a hydrophobic core in the dyes leads to fluorescence
quenching, the addition of a biocompatible organic solvent, such as

1-50% dimethylsulfoxide (DMSO) for example, restored fluorescence by preventing aggregation and allowed *in vivo* organ visualization.

The inventive dye-biomolecule conjugates are used for optical tomographic, endoscopic, photoacoustic and sonofluorescent applications

5 for the detection and treatment of tumors and other abnormalities.

Dye-biomolecule conjugates are also used for localized therapy. This may be accomplished by attaching a porphyrin or other photodynamic therapy agent to a bioconjugate, shining light of an appropriate wavelength to activate the agent, and detecting and/or

10 treating the abnormality.

The inventive conjugates can also be used for the detection of the presence of tumors and other abnormalities by monitoring the blood clearance profile of the conjugates, for laser assisted guided surgery for the detection of small micrometastases of, e.g., somatostatin subtype 2

15 (SST-2) positive tumors, upon laparoscopy, and for diagnosis of atherosclerotic plaques and blood clots.

The compositions of the invention can be formulated into diagnostic and therapeutic compositions for enteral or parenteral administration. These compositions contain an effective amount of the

20 dye along with conventional pharmaceutical carriers and excipients appropriate for the type of administration contemplated. For example, parenteral formulations advantageously contain the inventive agent in a sterile aqueous solution or suspension. Parenteral compositions may be

injected directly or mixed with a large volume parenteral composition for systemic administration. Such solutions also may contain pharmaceutically acceptable buffers and, optionally, electrolytes such as sodium chloride.

5 Formulations for enteral administration may vary widely, as is well known in the art. In general, such formulations are liquids, which include an effective amount of the inventive agent in aqueous solution or suspension. Such enteral compositions may optionally include buffers, surfactants, thixotropic agents, and the like. Compositions for oral
10 administration may also contain flavoring agents and other ingredients for enhancing their organoleptic qualities.

 The diagnostic compositions are administered in doses effective to achieve the desired enhancement. Such doses may vary widely, depending upon the particular dye employed, the organs or tissues
15 to be imaged, the imaging equipment being used, and the like. The diagnostic compositions of the invention are used in the conventional manner. The compositions may be administered to a patient, typically a warm-blooded animal, either systemically or locally to the organ or tissue to be imaged, and the patient is then subjected to the imaging procedure.

20 The inventive compositions and methods represent an important approach to the synthesis and use of novel cyanine and indocyanine dyes with a variety of photophysical and chemical properties.

The combination also represents an important approach to the use of small molecular targeting groups to image tumors by optical methods. The invention is further detailed in the following Examples, which are offered by way of illustration and are not intended to limit the scope of the invention in any manner.

EXAMPLE 1

Synthesis of Bis(ethylcarboxymethyl)indocyanine Dye (FIG. 1, $R_1, R_2 =$ fused phenyl; $A = CH_2, n = 1$ and $R = R' = CO_2H$)

10 A mixture of 1,1,2-trimethyl-[1H]-benz[e]indole (9.1 g, 43.58 mmols) and 3-bromopropanoic acid (10.0 g, 65.37 mmols) in 1,2-dichlorobenzene (40 mL) was heated at 110°C for 12 hours. The solution was cooled to room temperature and the red residue obtained was filtered and washed with acetonitrile:diethyl ether (1:1) mixture. The solid
15 obtained was dried under vacuum to give 10 g (64%) of light brown powder. A portion of this solid (6.0 g; 16.56 mmols), glutaconaldehyde dianil monohydrochloride (2.36 g, 8.28 mmols), and sodium acetate trihydrate (2.93 g, 21.53 mmols) in ethanol (150 mL) were refluxed for
20 90 minutes. After evaporating the solvent, 40 mL of 2 N aqueous HCl was added to the residue. The mixture was centrifuged and the supernatant was decanted. This procedure was repeated until the supernatant became nearly colorless. About 5 mL of water:acetonitrile (3:2) mixture was added to the solid residue and lyophilized to obtain 2 g

of dark green flakes. The purity of the compound was established with ^1H -NMR and liquid chromatography-mass spectroscopy (LC-MS).

EXAMPLE 2

5 Synthesis of Bis(pentylcarboxymethyl)indocyanine Dye (FIG. 1, $R_1, R_2 =$ fused phenyl; $A = \text{CH}_2$, $n = 4$ and $R = R' = \text{CO}_2\text{H}$)

A mixture of 1,1,2-trimethyl-[1H]-benz[e]indole (20 g, 95.6
mmoles) and 6-bromohexanoic acid (28.1 g, 144.1 mmoles) in 1,2-
10 dichlorobenzene (250 mL) was heated at 110°C for 12 hours. The green
solution was cooled to room temperature and the brown solid precipitate
formed was collected by filtration. After washing the solid with 1,2-
dichlorobenzene and diethyl ether, the brown powder obtained (24 g,
64%) was dried under vacuum at room temperature. A portion of this
15 solid (4.0 g; 9.8 mmoles), glutaconaldehyde dianil monohydrochloride (1.4
g, 5 mmoles) and sodium acetate trihydrate (1.8 g, 12.9 mmoles) in
ethanol (80 mL) were refluxed for 1 hour. After evaporating the solvent,
20 mL of 2 N aqueous HCl was added to the residue. The mixture was
centrifuged and the supernatant was decanted. This procedure was
20 repeated until the supernatant became nearly colorless. About 5 mL of
water:acetonitrile (3:2) mixture was added to the solid residue and
lyophilized to obtain about 2 g of dark green flakes. The purity of the
compound was established with ^1H -NMR and LC-MS.

EXAMPLE 3

Synthesis of Bisethylcarboxymethylindocyanine Dye (FIG. 1, $R_1 = R_2 = H$; $A = CH_2$, $n = 1$ and $R = R' = CO_2H$)

- 5 This compound was prepared as described in Example 1 except that 1,1,2-trimethylindole was used as the starting material.

EXAMPLE 4

- 10 Synthesis of Bis(hexaethyleneglycolcarboxymethyl)indocyanine Dye (FIG. 1, $R_1 = R_2 =$ fused phenyl; $A = CH_2OCH_2$, $n = 6$ and $R = R' = CO_2H$)

- This compound was prepared as described in Example 1 except that ω-bromohexaoxyethyleneglycolpropionic acid was used in place of bromopropanoic acid and the reaction was carried out in 1,2-dimethoxypropane.
- 15

EXAMPLE 5

- 20 Synthesis of Bisethylcarboxymethylindocyanine Dye (FIG. 2, $R_1 = R_2 =$ fused phenyl; $A = CH_2$, and $n = 0$)

- A solution of 50 ml of dimethylformamide and benzyl bromoacetate (16.0 g, 70 mmol) was stirred in a 100 ml three-neck flask. Solid potassium bicarbonate (7.8 g, 78 mmol) was added. The flask was purged with argon and cooled to 0°C with an ice bath. To the stirring mixture was added dropwise a solution of ethanolamine (1.9 g, 31 mmol) and 4 ml of dimethylformamide over 5 minutes. After the addition was complete the mixture was stirred for 1 hour at 0°C. The ice bath was removed and the mixture was stirred at room temperature overnight. The
- 25

reaction mixture was partitioned between 100 ml of methylene chloride and 100 ml of saturated sodium bicarbonate solution. The layers were separated and the methylene chloride layer was again washed with 100 ml of saturated sodium bicarbonate solution. The combined aqueous
5 layers were extracted twice with 25 ml of methylene chloride. The combined methylene chloride layers were washed with 100 ml of brine, and dried over magnesium sulfate. The methylene chloride was removed with aspirator vacuum at about 35°C, and the remaining dimethylformamide was removed with vacuum at about 45°C. The crude
10 material was left on a vacuum line overnight at room temperature.

The crude material was then dissolved in 100 ml of methylene chloride at room temperature. Triphenylphosphine (8.91 g, 34 mmol) was added and dissolved with stirring. An argon purge was started and the mixture was cooled to 0°C with an ice bath. The
15 N-bromosuccinimide (6.05 g, 34 mmol) was added portionwise over two minutes. The mixture was stirred for 1.5 hours at 0°C. The methylene chloride was removed with vacuum and gave a purple oil. This oil was triturated with 200 ml of ether with constant manual stirring. During this time the oil became very thick. The ether solution was decanted and the
20 oil was triturated with 100 ml of ether. The ether solution was decanted and the oil was again triturated with a 100 ml portion of ether. The ether was decanted and the combined ether solution was allowed to stand for about two hours to allow the triphenylphosphine oxide to crystallize. The

ether solution was decanted from the crystals and the solid was washed with 100 ml of ether. The volume of the combined ether extracts was reduced with vacuum until a volume of about 25 ml was obtained. This was allowed to stand overnight at 0°C. Ether (10 ml) was added to the
5 cold mixture, which was mixed to suspend the solid. The mixture was percolated through a column of 45 g of silica gel and eluted with ether, and 75 ml fractions were collected. The fractions that contained product, as determined by thin layer chromatography, were pooled and the ether was removed with vacuum. This yielded 10.1 g of crude product. The
10 material was flash chromatographed on silica gel with hexane, changing to 9:1 hexane:ether. The product-containing fractions were pooled and the solvents removed with vacuum. This yielded 7.4 g (57% yield) of pure product.

A mixture of 10% palladium on carbon (1 g) and a solution
15 of the benzyl ester (10 g) in 150 ml of methanol was hydrogenolyzed at 25 psi for two hours. The mixture was filtered over celite and the residue was washed with methanol. The solvent was evaporated to give a viscous oil in quantitative yield.

Reaction of the bromide with 1,1,2-trimethyl-[1H]-
20 benz[e]indole was carried out as described in Example 1.

EXAMPLE 6

Bis(ethylcarboxymethyldihydroxyl)indocyanine Dye (FIG. 3)

The hydroxy-indole compound is readily prepared by a known method (P. L. Southwick et al., One pot Fischer synthesis of (2,3,3-trimethyl-3-H-indol-5-yl)-acetic acid derivatives as intermediates for fluorescent biolabels. *Org. Prep. Proced. Int. Briefs*, 1988, 20(3), 279-284). Reaction of p-carboxymethylphenylhydrazine hydrochloride (30 mmol, 1 equiv.) and 1,1-bis(hydroxymethyl)propanone (45 mmole, 1.5 equiv.) in acetic acid (50 mL) at room temperature for 30 minutes and at
5
10
15
reflux for one minute gives (3,3-dihydroxymethyl-2-methyl-3-H-indol-5-yl)-acetic acid as a solid residue. The reaction of 3-bromopropyl-N,N-bis(carboxymethyl)amine, which was prepared as described in Example 5, with the intermediate indole and subsequent reaction of the indole intermediate with glutaconaldehyde dianil monohydrochloride (see Example 1) gives the desired product.

EXAMPLE 7

Synthesis of Bis(propylcarboxymethyl)indocyanine Dye (FIG. 4)

The intermediate 2-chloro-1-formyl-3-hydroxymethylenecyclohexane was prepared as described in the literature (G. A. Reynolds and K. H. Drexhage, Stable heptamethine pyrylium dyes that absorb in the infrared. *J. Org. Chem.*, 1977, 42(5), 885-888). Equal
20
volumes (40 mL each) of dimethylformamide (DMF) and dichloromethane

were mixed and the solution was cooled to -10°C in an acetone-dry ice bath. Under argon atmosphere, phosphorus oxychloride (40 mL) in dichloromethane was added dropwise to the cool DMF solution, followed by the addition of 10 g of cyclohexanone. The resulting solution was
5 allowed to warm to room temperature and was refluxed for six hours. After cooling to room temperature, the mixture was poured into ice-cold water and stored at 4°C for twelve hours. About 8 g of yellow powder was obtained after filtration. Condensation of the cyclic dialdehyde with the indole intermediate is carried out as described in Example 1. Further
10 functionalization of the dye with bis isopropylidene acetal protected monosaccharide was accomplished by the method described in the literature (J. H. Flanagan, et al., Near infrared heavy-atom-modified fluorescent dyes for base-calling in DNA-sequencing application using temporal discrimination. *Anal. Chem.*, 1998, 70(13), 2676-2684).

15

EXAMPLE 8

Synthesis of Bis(ethylcarboxymethyl)indocyanine Dye (FIG. 5)

These dyes are prepared as described in Example 7. These dyes absorb in the infrared region. The typical example shown in FIG. 5
20 has an estimated absorption maximum at 1036 nm.

EXAMPLE 9

Synthesis of Peptides

The procedure described below is for the synthesis of Octreotate. The amino acid sequence of Octreotate is: D-Phe-Cys'-Tyr-D-Trp-Lys-Thr-Cys'-Thr (SEQ ID NO:1), wherein Cys' indicates the presence of an intramolecular disulfide bond between two cysteine amino acids. Other peptides of this invention were prepared by a similar procedure with slight modifications in some cases.

The octapeptide was prepared by an automated
10 fluorenylmethoxycarbonyl (Fmoc) solid phase peptide synthesis using a commercial peptide synthesizer from Applied Biosystems (Model 432A SYNERGY Peptide Synthesizer). The first peptide cartridge contained Wang resin pre-loaded with Fmoc-Thr on 25 μ mole scale. Subsequent cartridges contained Fmoc-protected amino acids with side chain
15 protecting groups for the following amino acids: Cys(Acm), Thr(t-Bu), Lys(Boc), Trp(Boc) and Tyr(t-Bu). The amino acid cartridges were placed on the peptide synthesizer and the product was synthesized from the C- to the N-terminal position. The coupling reaction was carried out with 75 μ moles of the protected amino acids in the presence of
20 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/N-hydroxybenzotriazole (HOBt). The Fmoc protecting group was removed with 20% piperidine in dimethylformamide. After the synthesis was complete, the thiol group was cyclized with thallium trifluoroacetate

and the product was cleaved from the solid support with a cleavage mixture containing trifluoroacetic acid (85%):water (5%):phenol (5%):thioanisole (5%) for six hours. The peptide was precipitated with t-butyl methyl ether and lyophilized with water:acetonitrile (2:3) mixture.

- 5 The peptide was purified by HPLC and analyzed with LC/MS.

Octreotide, D-Phe-Cys'-Tyr-D-Trp-Lys-Thr-Cys'-Thr-OH (SEQ ID NO:2), wherein Cys' indicates the presence of an intramolecular disulfide bond between two cysteine amino acids, was prepared by the same procedure.

- 10 Bombesin analogs were prepared by the same procedure except that cyclization with thallium trifluoroacetate was not needed. Side-chain deprotection and cleavage from the resin was carried out with 50 μ L each of ethanedithiol, thioanisole and water, and 850 μ L of trifluoroacetic acid. Two analogues were prepared: Gly-Ser-Gly-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH₂ (SEQ ID NO:3) and Gly-Asp-Gly-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH₂ (SEQ ID NO:4).
- 15

- Cholecystokinin octapeptide analogs were prepared as described for Octreotate without the cyclization step. Three analogs were prepared: Asp-Tyr-Met-Gly-Trp-Met-Asp-Phe-NH₂ (SEQ ID NO:5); Asp-Tyr-Nle-Gly-Trp-Nle-Asp-Phe-NH₂ (SEQ ID NO:6); and D-Asp-Tyr-Nle-Gly-Trp-Nle-Asp-Phe-NH₂ (SEQ ID NO:7), where Nle is norleucine.
- 20

A neurotensin analog, D-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu (SEQ ID NO:8), was prepared as described for Octreotate without the cyclization step.

5

EXAMPLE 10

Synthesis of Peptide-Dye Conjugates (FIG. 6)

The method described below is for the synthesis of Octreotate-cyanine dye conjugates, but a similar procedure is used for the synthesis of other peptide-dye conjugates.

10

Octreotate was prepared as described in Example 9 but the peptide was not cleaved from the solid support and the N-terminal Fmoc group of Phe was retained. The thiol group was cyclized with thallium trifluoroacetate and the Phe was deprotected to liberate the free amine. Bisethylcarboxymethylindocyanine dye (53 mg, 75 μ moles) was added to an activation reagent consisting of a 0.2 M solution of HBTU/HOBt in DMSO (375 μ L), and 0.2 M solution of diisopropylethylamine in DMSO (375 μ L). The activation was complete in about 30 minutes and the resin-bound peptide (25 μ moles) was added to the dye. The coupling reaction was carried out at room temperature for three hours. The mixture was filtered and the solid residue was washed with DMF, acetonitrile and THF. After drying the green residue, the peptide was cleaved from the resin and the side chain protecting groups were removed with a mixture of 85% trifluoroacetic acid, 2.5% water, 2.5% thioanisole

15

20

and 2.5% phenol. The resin was filtered and cold t-butyl methyl ether (MTBE) was used to precipitate the dye-peptide conjugate, which was dissolved in an acetonitrile:water (2:3) mixture and lyophilized. The product was purified by HPLC to give the monoOctreotate-

- 5 Bisethylcarboxymethylindocyanine dye (Cytate 1, 80%) and the bisOctreotate-Bisethylcarboxymethylindocyanine dye (Cytate 2, 20%). The monoOctreotate conjugate is obtained almost exclusively (>95%) over the bis conjugate by reducing the reaction time to two hours. However, this also leads to incomplete reaction, and the free Octreotate
10 must be carefully separated from the dye conjugate in order to avoid saturation of the receptors by the non-dye conjugated peptide.

Octreotate-bispenitylcarboxymethylindocyanine dye was prepared as described above with some modifications.

- Bispenitylcarboxymethylindocyanine dye (60 mg, 75 μ moles) was added to
15 an activation reagent consisting of a 0.2 M solution of HBTU/HOBt in DMSO (400 μ L), and a 0.2 M solution of diisopropylethylamine in DMSO (400 μ L). The activation was complete in about 30 minutes and the resin-bound peptide (25 μ moles) was added to the dye. The reaction was carried out at room temperature for three hours. The mixture was filtered
20 and the solid residue was washed with DMF, acetonitrile and THF. After drying the green residue, the peptide was cleaved from the resin and the side chain protecting groups were removed with a mixture of 85% trifluoroacetic acid, 2.5% water, 2.5% thioanisole and 2.5% phenol. The

resin was filtered and cold t-butyl methyl ether (MTBE) was used to precipitate the dye-peptide conjugate, which was dissolved in an acetonitrile:water (2:3) mixture and lyophilized. The product was purified by HPLC to give Octreotate-1,1,2-trimethyl-[1H]-benz[e]indole propanoic acid conjugate (10%), monoOctreotate-bispentylcarboxymethylindocyanine dye (Cytate 3, 60%) and bisOctreotate-bispentylcarboxymethylindocyanine dye (Cytate 4, 30%).

EXAMPLE 11

10 Formulation of peptide-dye conjugates in dimethyl sulfoxide (DMSO)

The dye-peptide conjugates are sparingly soluble in water and require the addition of solubilizing agents or co-solvents. Addition of 1-20% aqueous ethanol to the conjugates partially quenched the fluorescence intensity *in vitro* and the fluorescence was completely quenched *in vivo* (the conjugate was not detected by the charge coupled device (CCD) camera). Addition of 1-50% of DMSO either re-established or increased the fluorescence intensity of the conjugates *in vitro* and *in vivo*. The dye fluorescence remained intense for over one week. The DMSO formulations were well tolerated by experimental animals used for this invention.

EXAMPLE 12

Imaging of pancreatic ductal adenocarcinoma (DSL 6A) with Indocyanine Green (ICG)

5 A non-invasive *in vivo* fluorescence imaging apparatus was employed to assess the efficacy of contrast agents developed for tumor detection in animal models. A LaserMax Inc. laser diode of nominal wavelength 780 nm and nominal power of 40 mW was used. The detector was a Princeton Instruments model RTE/CCD-1317-K/2 CCD
10 camera with a Rodenstock 10 mm F2 lens (stock #542.032.002.20) attached. An 830 nm interference lens (CVI Laser Corp., part # F10-830-4-2) was mounted in front of the CCD input lens such that only emitted fluorescent light from the contrast agent was imaged. Typically, an image of the animal was taken pre-injection of contrast agent. This image was
15 subsequently subtracted (pixel by pixel) from the post injection images. However, the background subtraction was never done once the animal had been removed from the sample area and returned at a later time for images taken several hours post injection.

 DSL 6A tumors were induced in male Lewis rats in the left
20 flank area by the introduction of material from a solid (donor) implant, and the tumors were palpable in approximately 14 days. The animals were anesthetized with xylazine: ketamine: acepromazine, 1.5: 1.5: 0.5 at 0.8 mL/kg via intramuscular injection. The area of the tumor (left flank) was shaved to expose the tumor and the surrounding surface area. A 21
25 gauge butterfly equipped with a stopcock and two syringes containing

FIGS. 7A-B are tumor images at two minutes (FIG. 7A) and 30 minutes (FIG. 7B) post bolus injection of a 0.5 mL aqueous solution of ICG (5.4 μ m). Tetracarboxylic acid cyanine dyes were synthesized as shown in FIG. 2, with A = CH₂ or CH₂OCH₂; R₁ = R₂ = H (Formula 1) or R₁, R₂ = fused phenyl (Formula 2).

15 EXAMPLE 13

Imaging of Prostatic Carcinoma (R3327-H) with Indocyanine Green (ICG)

The imaging apparatus and the procedure used are described as in Example 12. Prostate tumors (Dunning R3327-H) were induced in young male Copenhagen rats in the left flank area from a solid implant. These tumors grow very slowly and palpable masses were present 4-5 months post implant. FIGS. 7C-D are images of a rat with an induced prostatic carcinoma tumor (R3327-H) imaged at two minutes (FIG. 7C) and 30 minutes (FIG. 7D) post injection.

The Figures are false color images of fluorescent intensity measured at the indicated times, with images constrained to the tumor and a small surrounding area. As is shown, the dye intensity in the tumor is considerably diminished 30 minutes post-ICG injection.

5

EXAMPLE 14

Imaging of Rat Pancreatic Acinar Carcinoma (CA20948) with Indocyanine Green (ICG)

10 The imaging apparatus and the procedure used are described in Example 12. Rat pancreatic acinar carcinoma expressing the SST-2 receptor (CA20948) was induced by solid implant technique in the left flank area, and palpable masses were detected nine days post implant. The images obtained at 2 and 30 minutes post injection are shown in
15 FIG. 7E-F. FIGS. 7E-F are images of a rat with an induced pancreatic acinar carcinoma (CA20948) expressing the SST-2 receptor imaged at two minutes (FIG. 7E) and 30 minutes (FIG. 7F) post injection.

 The Figures are false color images of fluorescent intensity measured at the indicated times, with images constrained to the tumor
20 and a small surrounding area. As is shown, the dye intensity in the tumor is considerably diminished and almost absent 30 minutes post-ICG injection.

EXAMPLE 15

Imaging of Rat Pancreatic Acinar Carcinoma (CA20948) with Cytate 1

The imaging apparatus and the procedure used are described in Example 12, except that each animal received 500 μ l of a 1.0 mg/mL solution of Cytate 1 solution of 25% dimethylsulfoxide in water.

Rat pancreatic acinar carcinoma expressing the SST-2 receptor (CA20948) were induced by solid implant technique in the left flank area, and palpable masses were detected 24 days post implant. Images were obtained at various times post injection. Uptake into the tumor was seen at two minutes but was not maximal until about five minutes.

FIGS. 8A-B show a comparison of the uptake of ICG and Cytate 1 at 45 minutes in rats with the CA20948 tumor cell line. By 45 minutes the ICG has mostly cleared (FIG. 8A) whereas the Cytate 1 is still intense (FIG. 8B). This dye fluorescence remained intense in the tumor for several hours post-injection.

EXAMPLE 16

Imaging of Rat Pancreatic Acinar Carcinoma (CA20948) with Cytate 1 Compared with Imaging with Indocyanine Green

Using indocyanine green (ICG), three different tumor lines were imaged optically using a CCD camera apparatus. Two of the lines, DSL 6/A (pancreatic) and Dunning R3327H (prostate) indicated slow perfusion of the agent over time into the tumor and reasonable images

were obtained for each. The third line, CA20948 (pancreatic), indicated only a slight but transient perfusion that was absent after only 30 minutes post injection. This indicated no non-specific localization of ICG into this line compared to the other two tumor lines, suggesting a different vascular architecture for this type of tumor (see FIGS. 7A-F). The first two tumor lines (DSL 6/A and R3327H) are not as highly vascularized as CA20948, which is also rich in somatostatin (SST-2) receptors. Consequently, the detection and retention of a dye in this tumor model is a good index of receptor-mediated specificity.

10 Octreotate is known to target somatostatin (SST-2) receptors, hence, cyano-Octreotates (Cytate 1 and Cytate 2) were prepared. Cytate 1 was evaluated in the CA20948 Lewis rat model. Using the CCD camera apparatus, localization of this dye was observed in the tumor (indicated by arrow) at 45 minutes post injection (FIG. 9A). At 15 27 hours post injection, the animal was again imaged (FIG. 9B). Tumor visualization was easily observed (indicated by arrow), showing specificity of this agent for the SST-2 receptors present in the CA20948 tumor line.

Individual organs were removed at about 24 hours post Cytate 1 administration and imaged. As shown in FIG. 10, high uptake of Cytate 1 was observed in the pancreas, adrenals and tumor tissue, while heart, muscle, spleen and liver indicated significantly lower uptake. These data correlate well with radiolabeled Octreotate in the same model system (M. de Jong, et al. *Cancer Res.* 1998, 58, 437-441).

EXAMPLE 17

Imaging of Rat Pancreatic Acinar Carcinoma (AR42-J) with bombesinate

The AR42-J cell line is derived from exocrine rat pancreatic
5 acinar carcinoma. It can be grown in continuous culture or maintained *in*
vivo in athymic nude mice, SCID mice, or in Lewis rats. This cell line is
particularly attractive for *in vitro* receptor assays, as it is known to
express a variety of hormone receptors including cholecystokinin (CCK),
epidermal growth factor (EGF), pituitary adenylate cyclase activating
10 peptide (PACAP), somatostatin (SST-2) and bombesin.

In this model, male Lewis rats were implanted with solid
tumor material in a similar manner as described for the CA20948 rat
model. Palpable masses were present seven days post implant, and
imaging studies were conducted on animals at 10-12 days post implant
15 when the mass had achieved about 2-2.5 g.

FIG. 11 is an image of bombesinate in an AR42-J tumor-
bearing rat, as described in Example 16, at 22 hours post injection of
bombesinate. As shown in FIG. 11, specific localization of the
bioconjugate in the tumor (indicated by arrow) was observed.

20

EXAMPLE 18

Monitoring of the blood clearance profile of peptide-dye conjugates

A laser of appropriate wavelength for excitation of the dye
chromophore was directed into one end of a fiber optic bundle and the

other end was positioned a few millimeters from the ear of a rat. A second fiber optic bundle was also positioned near the same ear to detect the emitted fluorescent light and the other end was directed into the optics and electronics for data collection. An interference filter (IF) in the collection optics train was used to select emitted fluorescent light of the appropriate wavelength for the dye chromophore.

Sprague-Dawley or Fischer 344 rats were used in these studies. The animals were anesthetized with urethane administered via intraperitoneal injection at a dose of 1.35 g/kg body weight. After the animals had achieved the desired plane of anesthesia, a 21 gauge butterfly with 12" tubing was placed in the lateral tail vein of each animal and flushed with heparinized saline. The animals were placed on a heating pad and kept warm throughout the entire study. The lobe of the left ear was affixed to a glass microscope slide to reduce movement and vibration.

Incident laser light delivered from the fiber optic was centered on the affixed ear. Data acquisition was then initiated, and a background reading of fluorescence was obtained prior to administration of the test agent. For Cytates 1 or 2, the peptide-dye conjugate was administered to the animal through a bolus injection, typically 0.5 to 2.0 mL, in the lateral tail vein. This procedure was repeated with several dye-peptide conjugates in normal and tumor-bearing rats. Representative profiles as a method to monitor blood clearance of the peptide-dye

conjugate in normal and tumor-bearing animals are shown in FIGS. 12 to 16. The data were analyzed using a standard sigma plot software program for a one-compartment model.

In rats treated with Cytates 1 or 2, the fluorescence signal rapidly increased to a peak value. The signal then decayed as a function of time as the conjugate cleared from the bloodstream. FIG. 12 shows the clearance profile of Cytate 1 from the blood of a normal rat monitored at 830 nm after excitation at 780 nm. FIG. 13 shows the clearance profile of Cytate 1 from the blood of a pancreatic tumor (CA20948)-bearing rat also monitored at 830 nm after excitation at 780 nm.

FIG. 14 shows the clearance profile of Cytate 2 from the blood of a normal rat, and FIG. 15 shows the clearance profile of Cytate 2 from the blood of a pancreatic tumor (CA20948)-bearing rat, monitored at 830 nm after excitation at 780 nm.

FIG. 16 shows the clearance profile of Cytate 4 from the blood of a normal rat monitored at 830 nm after excitation at 780 nm.

It should be understood that the embodiments of the present invention shown and described in the specification are only specific embodiments of the inventors who are skilled in the art and are not limiting in any way. Therefore, various changes, modifications or alterations to those embodiments may be made or resorted to without departing from the spirit of the invention and the scope of the following

[illegible]

SEQUENCE LISTING

<110> APPLICANT: Samuel I. Achilefu
Raghavan Rajagopalan
Richard B. Dorshow
Joseph E. Bugaj
ASSIGNEE: Mallinckrodt Inc.

<120> TITLE: Hydrophilic Cyanine Dyes

<130> DOCKET/FILE REFERENCE: MRD-66

<150> PRIOR APPLICATION NUMBER: 09/484,319

<151> FILING DATE: 2000-01-18

<160> NUMBER OF SEQUENCES: 8

<170> SOFTWARE: FastSEQ for Windows Version 3.0

<210> SEQ ID NO:1

<211> LENGTH: 8

<212> TYPE: PRT

<213> ORGANISM:Synthetic

<221> MOD_RES

<222> (1)...(0)

<223> Xaa = D-Phe

<224> Xbb = Cys with an intramolecular disulfide bond
between two Cys amino acids

<225> Xcc = D-Trp

<400> SEQ ID NO:1

Xaa Xbb Tyr Xcc Lys Thr Xbb Thr
1 5

<210> SEQ ID NO:2

<211> LENGTH: 8

<212> TYPE: PRT

<213> ORGANISM:Synthetic

<221> MOD_RES

<222> (1)...(0)

<223> Xaa = D-Phe

<224> Xbb = Cys with an intramolecular disulfide bond
between two Cys amino acids

<225> Xcc = D-Trp

<226> Xdd = Thr-OH

<400> SEQ ID NO:2

Xaa Xbb Tyr Xcc Lys Thr Xbb Xdd
1 5

```

      <400> SEQ ID NO:6
Asp Tyr Nle Gly Trp Nle Asp Phe
 1               5

```


<210> SEQ ID NO:7
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM:Synthetic

<221> MOD_RES
<222> (1)...(0)

<228> Xff = D-Asp

<400> SEQ ID NO:7
Xff Tyr Nle Gly Trp Nle Asp Phe
1 5

<210> SEQ ID NO:8
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM:Synthetic

<229> Xgg = D-Lys

<400> SEQ ID NO:8
Xgg Pro Arg Arg Pro Tyr Ile Leu
1 5

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
66
67
68
69
70
71
72
73
74
75
76
77
78
79
80
81
82
83
84
85
86
87
88
89
90
91
92
93
94
95
96
97
98
99
100
101
102
103
104
105
106
107
108
109
110
111
112
113
114
115
116
117
118
119
120
121
122
123
124
125
126
127
128
129
130
131
132
133
134
135
136
137
138
139
140
141
142
143
144
145
146
147
148
149
150
151
152
153
154
155
156
157
158
159
160
161
162
163
164
165
166
167
168
169
170
171
172
173
174
175
176
177
178
179
180
181
182
183
184
185
186
187
188
189
190
191
192
193
194
195
196
197
198
199
200
201
202
203
204
205
206
207
208
209
210
211
212
213
214
215
216
217
218
219
220
221
222
223
224
225
226
227
228
229
230
231
232
233
234
235
236
237
238
239
240
241
242
243
244
245
246
247
248
249
250
251
252
253
254
255
256
257
258
259
260
261
262
263
264
265
266
267
268
269
270
271
272
273
274
275
276
277
278
279
280
281
282
283
284
285
286
287
288
289
290
291
292
293
294
295
296
297
298
299
300
301
302
303
304
305
306
307
308
309
310
311
312
313
314
315
316
317
318
319
320
321
322
323
324
325
326
327
328
329
330
331
332
333
334
335
336
337
338
339
340
341
342
343
344
345
346
347
348
349
350
351
352
353
354
355
356
357
358
359
360
361
362
363
364
365
366
367
368
369
370
371
372
373
374
375
376
377
378
379
380
381
382
383
384
385
386
387
388
389
390
391
392
393
394
395
396
397
398
399
400
401
402
403
404
405
406
407
408
409
410
411
412
413
414
415
416
417
418
419
420
421
422
423
424
425
426
427
428
429
430
431
432
433
434
435
436
437
438
439
440
441
442
443
444
445
446
447
448
449
450
451
452
453
454
455
456
457
458
459
460
461
462
463
464
465
466
467
468
469
470
471
472
473
474
475
476
477
478
479
480
481
482
483
484
485
486
487
488
489
490
491
492
493
494
495
496
497
498
499
500
501
502
503
504
505
506
507
508
509
510
511
512
513
514
515
516
517
518
519
520
521
522
523
524
525
526
527
528
529
530
531
532
533
534
535
536
537
538
539
540
541
542
543
544
545
546
547
548
549
550
551
552
553
554
555
556
557
558
559
560
561
562
563
564
565
566
567
568
569
570
571
572
573
574
575
576
577
578
579
580
581
582
583
584
585
586
587
588
589
590
591
592
593
594
595
596
597
598
599
600
601
602
603
604
605
606
607
608
609
610
611
612
613
614
615
616
617
618
619
620
621
622
623
624
625
626
627
628
629
630
631
632
633
634
635
636
637
638
639
640
641
642
643
644
645
646
647
648
649
650
651
652
653
654
655
656
657
658
659
660
661
662
663
664
665
666
667
668
669
670
671
672
673
674
675
676
677
678
679
680
681
682
683
684
685
686
687
688
689
690
691
692
693
694
695
696
697
698
699
700
701
702
703
704
705
706
707
708
709
710
711
712
713
714
715
716
717
718
719
720
721
722
723
724
725
726
727
728
729
730
731
732
733
734
735
736
737
738
739
740
741
742
743
744
745
746
747
748
749
750
751
752
753
754
755
756
757
758
759
760
761
762
763
764
765
766
767
768
769
770
771
772
773
774
775
776
777
778
779
780
781
782
783
784
785
786
787
788
789
790
791
792
793
794
795
796
797
798
799
800
801
802
803
804
805
806
807
808
809
810
811
812
813
814
815
816
817
818
819
820
821
822
823
824
825
826
827
828
829
830
831
832
833
834
835
836
837
838
839
840
841
842
843
844
845
846
847
848
849
850
851
852
853
854
855
856
857
858
859
860
861
862
863
864
865
866
867
868
869
870
871
872
873
874
875
876
877
878
879
880
881
882
883
884
885
886
887
888
889
890
891
892
893
894
895
896
897
898
899
900
901
902
903
904
905
906
907
908
909
910
911
912
913
914
915
916
917
918
919
920
921
922
923
924
925
926
927
928
929
930
931
932
933
934
935
936
937
938
939
940
941
942
943
944
945
946
947
948
949
950
951
952
953
954
955
956
957
958
959
960
961
962
963
964
965
966
967
968
969
970
971
972
973
974
975
976
977
978
979
980
981
982
983
984
985
986
987
988
989
990
991
992
993
994
995
996
997
998
999
1000
1001
1002
1003
1004
1005
1006
1007
1008
1009
1010
1011
1012
1013
1014
1015
1016
1017
1018
1019
1020
1021
1022
1023
1024
1025
1026
1027
1028
1029
1030
1031
1032
1033
1034
1035
1036
1037
1038
1039
1040
1041
1042
1043
1044
1045
1046
1047
1048
1049
1050
1051
1052
1053
1054
1055
1056
1057
1058
1059
1060
1061
1062
1063
1064
1065
1066
1067
1068
1069
1070
1071
1072
1073
1074
1075
1076
1077
1078
1079
1080
1081
1082
1083
1084
1085
1086
1087
1088
1089
1090
1091
1092
1093
1094
1095
1096
1097
1098
1099
1100
1101
1102
1103
1104
1105
1106
1107
1108
1109
1110
1111
1112
1113
1114
1115
1116
1117
1118
1119
1120
1121
1122
1123
1124
1125
1126
1127
1128
1129
1130
1131
1132
1133
1134
1135
1136
1137
1138
1139
1140
1141
1142
1143
1144
1145
1146
1147
1148
1149
1150
1151
1152
1153
1154
1155
1156
1157
1158
1159
1160
1161
1162
1163
1164
1165
1166
1167
1168
1169
1170
1171
1172
1173
1174
1175
1176
1177
1178
1179
1180
1181
1182
1183
1184
1185
1186
1187
1188
1189
1190
1191
1192
1193
1194
1195
1196
1197
1198
1199
1200
1201
1202
1203
1204
1205
1206
1207
1208
1209
1210
1211
1212
1213
1214
1215
1216
1217
1218
1219
1220
1221
1222
1223
1224
1225
1226
1227
1228
1229
1230
1231
1232
1233
1234
1235
1236
1237
1238
1239
1240
1241
1242
1243
1244
1245
1246
1247
1248
1249
1250
1251
1252
1253
1254
1255
1256
1257
1258
1259
1260
1261
1262
1263
1264
1265
1266
1267
1268
1269
1270
1271
1272
1273
1274
1275
1276
1277
1278
1279
1280
1281
1282
1283
1284
1285
1286
1287
1288
1289
1290
1291
1292
1293
1294
1295
1296
1297
1298
1299
1300
1301
1302
1303
1304
1305
1306
1307
1308
1309
1310
1311
1312
1313
1314
1315
1316
1317
1318
1319
1320
1321
1322
1323
1324
1325
1326
1327
1328
1329
1330
1331
1332
1333
1334
1335
1336
1337
1338
1339
1340
1341
1342
1343
1344
1345
1346
1347
1348
1349
1350
1351
1352
1353
1354
1355
1356
1357
1358
1359
1360
1361
1362
1363
1364
1365
1366
1367
1368
1369
1370
1371
1372
1373
1374
1375
1376
1377
1378
1379
1380
1381
1382
1383
1384
1385
1386
1387
1388
1389
1390
1391
1392
1393
1394
1395
1396
1397
1398
1399
1400
1401
1402
1403
1404
1405
1406
1407
1408
1409
1410
1411
1412
1413
1414
1415
1416
1417
1418
1419
1420
1421
1422
1423
1424
1425
1426
1427
1428
1429
1430
1431
1432
1433
1434
1435
1436
1437
1438
1439
1440
1441
1442
1443
1444
1445
1446
1447
1448
1449
1450
1451
1452
1453
1454
1455
1456
1457
1458
1459
1460
1461
1462
1463
1464
1465
1466
1467
1468
1469
1470
1471
1472
1473
1474
1475
1476
1477
1478
1479
1480
1481
1482
1483
1484
1485
1486
1487
1488
1489
1490
1491
1492
1493
1494
1495
1496
1497
1498
1499
1500
1501
1502
1503
1504
1505
1506
1507
1508
1509
1510
1511
1512
1513
1514
1515
1516
1517
1518
1519
1520
1521
1522
1523
1524
1525
1526
1527
1528
1529
1530
1531
1532
1533
1534
1535
1536
1537
1538
1539
1540
1541
1542
1543
1544
1545
1546
1547
1548
1549
1550
1551
1552
1553
1554
1555
1556
1557
1558
1559
1560
1561
1562
1563
1564
1565
1566
1567
1568
1569
1570
1571
1572
1573
1574
1575
1576
1577
1578
1579
1580
1581
1582
1583
1584
1585
1586
1587
1588
1589
1590
1591
1592
1593
1594
1595
1596
1597
1598
1599
1600
1601
1602
1603
1604
1605
1606
1607
1608
1609
1610
1611
1612
1613
1614
1615
1616
1617
1618
1619
1620
1621
1622
1623
1624
1625
1626
1627
1628
1629
1630
1631
1632
1633
1634
1635
1636
1637
1638
1639
1640
1641
1642
1643
1644
1645
1646
1647
1648
1649
1650
1651
1652
1653
1654
1655
1656
1657
1658
1659
1660
1661
1662
1663
1664
1665
1666
1667
1668
1669
1670
1671
1672
1673
1674
1675
1676
1677
1678
1679
1680
1681
1682
1683
1684
1685
1686
1687
1688
1689
1690
1691
1692
1693
1694
1695
1696
1697
1698
1699
1700
1701
1702
1703
1704
1705
1706
1707
1708
1709
1710
1711
1712
1713
1714
1715
1716
1717
1718
1719
1720
1721
1722
1723
1724
1725
1726
1727
1728
1729
1730
1731
1732
1733
1734
1735
1736
1737
1738
1739
1740
1741
1742
1743
1744
1745
1746
1747
1748
1749
1750
1751
1752
1753
1754
1755
1756
1757
1758
1759
1760
1761
1762
1763
1764
1765
1766
1767
1768
1769
1770
1771
1772
1773
1774
1775
1776
1777
1778
1779
1780
1781
1782
1783
1784
1785
1786
1787
1788
1789
1790
1791
1792
1793
1794
1795
1796
1797
1798
1799
1800
1801
1802
1803
1804
1805
1806
1807
1808
1809
1810
1811
1812
1813
1814
1815
1816
1817
1818
1819
1820
1821
1822
1823
1824
1825
1826
1827
1828
1829
1830
1831
1832
1833
1834
1835
1836
1837
1838
1839
1840
1841
1842
1843
1844
1845
1846
1847
1848
1849
1850
1851
1852
1853
1854
1855
1856
1857
1858
1859
1860
1861
1862
1863
1864
1865
1866
1867
1868
1869
1870
1871
1872
1873
1874
1875
1876
1877
1878
1879
1880
1881
1882
1883
1884
1885
1886
1887
1888
1889
1890
1891
1892
1893
1894
1895
1896
1897
1898
1899
1900
1901
1902
1903
1904
1905
1906
1907
1908
1909
1910
1911
1912
1913
1914
1915
1916
1917
1918
1919
1920
1921
1922
1923
1924
1925
1926
1927
1928
1929
1930
1931
1932
1933
1934
1935
1936
1937
1938
1939
1940
1941
1942
1943
1944
1945
1946
1947
1948
1949
1950
1951
1952
1953
1954
1955
1956
1957
1958
1959
1960
1961
1962
1963
1964
1965
1966
1967
1968
1969
1970
1971
1972
1973
1974
1975
1976
1977
1978
1979
1980
1981
1982
1983
1984
1985
1986
1987
1988
1989
1990
1991
1992
1993
1994
1995
1996
1997
1998
1999
2000
2001
2002
2003
2004
2005
2006
2007
2008
2009
2010
2011
2012
2013
2014
2015
2016
2017
2018
2019
2020
2021
2022
2023
2024
2025
2026
2027
2028
2029
2030
2031
2032
2033
2034
2035
2036
2037
2038
2039
2040
2041
2042
2043
2044
2045
2046
2047
2048
2049
2050
2051
2052
2053
2054
2055
2056
2057
2058
2059
2060
2061
2062
2063
2064
2065
2066
2067
2068
2069
2070
2071
2072
2073
2074
2075
2076
2077
2078
2079
2080
2081
2082
2083
2084
2085
2086
2087
2088
2089
2090
2091
2092
2093
2094
2095
2096
2097
2098
2099
2100
2101
2102
2103
2104
2105
2106
2107
2108
2109
2110
2111
2112
2113
2114
2115
2116
2117
2118
2119
2120
2121
2122
2123
2124
2125
2126
2127
2128
2129
2130
2131
2132
2133
2134
2135
2136
2137
2138
2139
2140
2141
2142
2143
2144
2145
2146
2147
2148
2149
2150
2151
2152
2153
2154
2155
2156
2157
2158
2159
2160
2161
2162
2163